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A high prevalence of multi-drug resistant Gram-negative bacilli in a Nepali tertiary care hospital and associated widespread distribution of Extended-Spectrum Beta-Lactamase (ESBL) and carbapenemase-encoding genes

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Abstract

Background: Multi-drug resistance (MDR) and extensive-drug resistance (XDR) associated with extended-spectrum beta-lactamases (ESBLs) and carbapenemases in Gram-negative bacteria are global public health concerns. Data on circulating antimicrobial resistance (AMR) genes in Gram-negative bacteria and their correlation with MDR and ESBL phenotypes from Nepal is scarce.

Methods: A retrospective study was performed investigating the distribution of ESBL and carbapenemase genes and their potential association with ESBL and MDR phenotypes in *E. coli*, *Klebsiella* spp., *Enterobacter* spp. and *Acinetobacter* spp. isolated in a major tertiary hospital in Kathmandu, Nepal, between 2012 and 2018.

Results: During this period, the hospital isolated 719 *E. coli*, 532 *Klebsiella* spp., 520 *Enterobacter* spp. and 382 *Acinetobacter* spp.; 1955/2153 (90.1%) of isolates were MDR and half (1080/2153) were ESBL producers. Upon PCR amplification, *bla*_{TEM} (1281/1771; 72%), *bla*_{CTX-M-1} (930/1771; 53%) and *bla*_{CTX-M-8} (419/1771; 24%) were the most prevalent ESBL genes in the enteric bacilli. *bla*_{OXA} and *bla*_{OXA-51} were the most common *bla*_{OXA} family genes in the enteric bacilli (918/1771; 25%) and *Acinetobacter* spp. (218/382; 57%) respectively. Sixteen percent (342/2153) of all isolates and 20% (357/1771) of enteric bacilli harboured *bla*_{NDM-1} and *bla*_{KPC} carbapenemase genes respectively. Of enteric bacilli, *Enterobacter* spp. was the most frequently positive for *bla*_{KPC} gene (201/337; 60%). The presence of each *bla*_{CTX-M} and *bla*_{OXA} were significantly associated with non-susceptibility to third generation cephalosporins (OR 14.7, $p < 0.001$ and OR 2.3, $p < 0.05$, respectively). The presence of each *bla*_{TEM}, *bla*_{CTX-M} and *bla*_{OXA} family genes were significantly associated with ESBL positivity (OR 2.96, $p < 0.001$; OR 14.2, $p < 0.001$ and OR 1.3, $p < 0.05$ respectively) and being MDR (OR 1.96, $p < 0.001$; OR 5.9, $p < 0.001$ and OR 2.3, $p < 0.001$ respectively).

Conclusions: This study documents an alarming level of AMR with high prevalence of MDR ESBL- and carbapenemase-positive ESKAPE microorganisms in our clinical setting. These data suggest a scenario where the clinical

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management of infected patients is increasingly difficult and requires the use of last-resort antimicrobials, which in turn is likely to intensify the magnitude of global AMR crisis.

Keyword: Multi-drug resistance, Antimicrobial resistance, Nepal, ESBL, Gram-negative bacilli, Carbapenemase, ESKAPE

Background

Gram-negative bacilli, particularly those in the bacterial family Enterobacteriaceae (e.g. *Klebsiella* spp. and *Enterobacter* spp.) and *Acinetobacter* spp. are common causes of serious community and hospital-acquired infections. These Gram-negative bacilli are also members of the ESKAPE group of pathogens [1]. These are notoriously associated with antimicrobial resistance (AMR) and frequently carry genes that induce resistance to three or more classes of antimicrobials, making them multi-drug resistant. Such multi-drug resistant Gram-negative bacilli represent a significant global public health problem as they are more commonly associated with worse outcomes than susceptible isolates [2–5]. The situation with multi-drug resistant Gram-negative bacilli is particularly alarming in South Asia, which is considered as a global epicentre of these microorganisms [6, 7].

Extended-spectrum beta-lactamases (ESBLs) are enzymes that can hydrolyse and therefore inactivate beta-lactam antimicrobials such as penicillins, cephalosporins and monobactams. ESBL activity is an important mechanism by which Gram-negative bacilli exhibit resistance against beta-lactam antimicrobials. Multiple ESBL variants have been detected and grouped into several structural and evolutionary families which include TEM, SHV, CTX-M, PER, VEB, GES, BES, TLA and OXA [8]. Of these, CTX-M and TEM are the most common ESBLs, while OXA enzymes such as OXA-48, 23, 24, 51, 58 are common carbapenemases that are associated with Gram-negative bacteria that cause nosocomial infections [8, 9].

The possession of ESBLs increases the risk of treatment failure with beta-lactam antimicrobials, may contribute to the spread of AMR in Gram-negative bacteria and may complicate infection control in hospital [9, 10]. Therefore, detection and reporting for the presence of ESBLs in bacterial pathogens are important for clinical care. This is also essential for developing optimal infection control measures in hospital [10]. Modified disc diffusion tests are routinely used to detect the presence of ESBLs and establish the nature of ESBL phenotypes [11]. Though being useful, these phenotypic detection methods are labour intensive and do not determine the classes of genes that are associated with ESBL activity. Molecular methods like PCR, DNA hybridization, or whole genome sequencing (WGS) may be used alongside phenotypic test methods to detect the presence of ESBL and

carbapenemase associated genes and generate additional information regarding the epidemiological distribution of key resistance genes in sentinel locations [10].

South Asia is a key location for antimicrobial resistant pathogens. A high prevalence of MDR and ESBL positive Enterobacteriaceae family bacteria and *Acinetobacter* have been reported across the region [12–19]. However, little is known about various ESBL and carbapenemase genes associated with these phenotypes in Gram-negative bacilli isolated in hospitals of Nepal. Furthermore, there are little data originating from this region on the association of these genotypes with the observed phenotypes. Such data are important for assessing the contribution of combined phenotypic and genetic tests on clinical care and providing better understanding of the impact of resistance genes on the epidemiology of circulating pathogens.

Here, aiming to better understand the epidemiology of multi-drug resistant microorganisms and improve clinical care in our healthcare facility, we determined the AMR profile of all *E. coli*, *Klebsiella* spp., *Enterobacter* spp. and *Acinetobacter* spp. isolated from in- and out-patients at a major tertiary hospital in Kathmandu, Nepal, between June 2012 and December 2018. We additionally screened them for the phenotypic presence of ESBL activity by disc diffusion method and performed PCR amplification to detect any ESBL and carbapenemase associated AMR genes. Lastly, we investigated the relationship between the presence of *bla*_{CTX-M}, *bla*_{TEM} and *bla*_{OXA} genes with an ESBL or MDR phenotype. Understanding the association between AMR genotype and phenotype is crucial to assess the value of genetic detection methods in clinical settings.

Methods

Study design

This was a retrospective study of anonymised routine microbiology laboratory results originating from Patan Hospital in Lalitpur metropolitan city of the Kathmandu Valley, Nepal. All data regarding *E. coli*, *Klebsiella* spp., *Enterobacter* spp. and *Acinetobacter* spp. that were isolated from June 2012 to December 2018 were included in this study. These data were devoid of any personal identification information as this work was performed as a component of routine surveillance for infection control at Patan Hospital.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed at the time of bacterial isolation by modified Kirby-Bauer disc diffusion method, as previously described [20]. Zone size interpretations were performed following the appropriate Clinical and Laboratory Standards Institute (CLSI) guidelines [21]. The antimicrobials against which organisms were tested are listed in Additional file 1: Table S1; not all isolates were tested for all antimicrobials.

The bacteria producing resistant or intermediate response against tested antimicrobials were grouped as “non-susceptible” for the purposes of analysis. MDR was defined as an acquired non-susceptibility (without intrinsic resistance) to at least one agent of three or more antimicrobial classes. Intrinsic resistance was defined according to the CLSI guideline of 2014 [21]. The following intrinsic resistance were reported but ignored for the purpose of generating MDR profiles: (i) *Acinetobacter* spp. resistant against amoxicillin or penicillin, (ii) *E. coli*, *Klebsiella*, *Enterobacter*, or *Acinetobacter* spp. against vancomycin, teicoplanin, erythromycin or azithromycin. Phenotypic testing for ESBL positivity was conducted using the combination disc diffusion method with a beta-lactam antimicrobial disc alone and that in combination with a beta-lactamase inhibitor (clavulanic acid) [22]. The isolate was considered as ESBL positive if the zone of inhibition around the beta-lactamase inhibitor supplemented disc was ≥ 5 mm in comparison to the respective beta-lactam antimicrobial alone. The products of Mast diagnostics (Mast group Ltd., Liverpool, UK) namely D62C and D68C were used in this study.

Detection of resistance genes

Bacterial DNA was extracted by suspending bacterial colonies in Phosphate buffered saline (PBS) and subjecting them to 100 °C for five minutes; the suspensions were centrifuged and the supernatant was used as template for PCR amplifications. The PCR amplifications were performed in multiplex following previously described primers and conditions. The ESBL targets were *bla*_{CTXM-1}, *bla*_{CTXM-2}, *bla*_{CTXM-8}, *bla*_{CTXM-9}, *bla*_{CTXM-25}, *bla*_{TEM} and *bla*_{SHV} [23]. The carbapenem resistance genes tested were *bla*_{OXA}, *bla*_{KPC}, *bla*_{OXA48} [24], *bla*_{NDM-1} [25], *bla*_{OXA1_4_30}, *bla*_{OXA23}, *bla*_{OXA24}, *bla*_{OXA51}, *bla*_{OXA58} [26], *bla*_{VIM} and *bla*_{IMP} [27].

For analysis purpose, the detection of any of *bla*_{OXA}, *bla*_{OXA1_4_30}, *bla*_{OXA23}, *bla*_{OXA24}, *bla*_{OXA48}, *bla*_{OXA51} or *bla*_{OXA58} was designated as *bla*_{OXA} positive. Similarly, the detection of any of *bla*_{CTXM-1}, *bla*_{CTXM-2}, *bla*_{CTXM-8}, *bla*_{CTXM-9} or *bla*_{CTXM-25} was designated as *bla*_{CTXM} positive. Fisher's exact test was used to test for an association between the detection of a resistance gene and a

specific resistance phenotype (ESBL positive, MDR, or resistance to third and fourth generation cephalosporins). All analyses were performed using the statistical software R version 3.5.3 and R Studio version 1.0.143. Venn diagrams were generated using the UpSetR R package [28].

Results

Bacterial isolates

Between June 2012 and December 2018, the microbiology laboratory at Patan hospital isolated 719 *E. coli*, 532 *Klebsiella* spp., 520 *Enterobacter* spp. and 383 *Acinetobacter* spp., totalling 2153 isolates (Table 1). A majority (512/2153; 23.8%) of isolates originated from the samples that were taken from the patients visiting emergency department followed by paediatric intensive care unit (PICU, 295/2153; 13.7%), gynaecology ward (287/2153; 13.3%) and general medical ward (208/2153; 9.7%). The distribution of ESBL-positivity and MDR in microorganisms by hospital departments is shown in Fig. 1. Half (1080/2153; 50.2%) of all bacterial isolates were ESBL positive (Table 2), which was predictably more common (> 50%) in *E. coli*, *Klebsiella* spp. and *Enterobacter* spp. isolates than in *Acinetobacter* spp. isolates (< 1%). Approximately 90% of *E. coli*, *Klebsiella* spp. and *Enterobacter* spp. were multi-drug resistant; this proportion was relatively lower in *Acinetobacter* spp. (82%).

Table 1 Summary of microorganisms

| | n | % |
|-------------------------------|------|------|
| Microorganisms (Total = 2153) | | |
| <i>E. coli</i> | 719 | 33.4 |
| <i>Klebsiella</i> spp. | 532 | 24.7 |
| <i>Enterobacter</i> spp. | 520 | 24.2 |
| <i>Acinetobacter</i> spp. | 382 | 17.7 |
| Departments (Total = 2153) | | |
| Emergency | 512 | 23.8 |
| Paediatric ICU | 295 | 13.7 |
| Gynaecology | 287 | 13.3 |
| Medical | 208 | 9.7 |
| Adult ICU | 154 | 7.2 |
| Nursery | 136 | 6.3 |
| Surgery | 116 | 5.4 |
| Paediatric | 125 | 5.8 |
| Not known | 219 | 10.2 |
| Outpatient | 82 | 3.8 |
| Orthopaedic | 19 | 0.9 |
| ESBL positive (Total = 2153) | | |
| No | 1073 | 49.8 |
| Yes | 1080 | 50.2 |

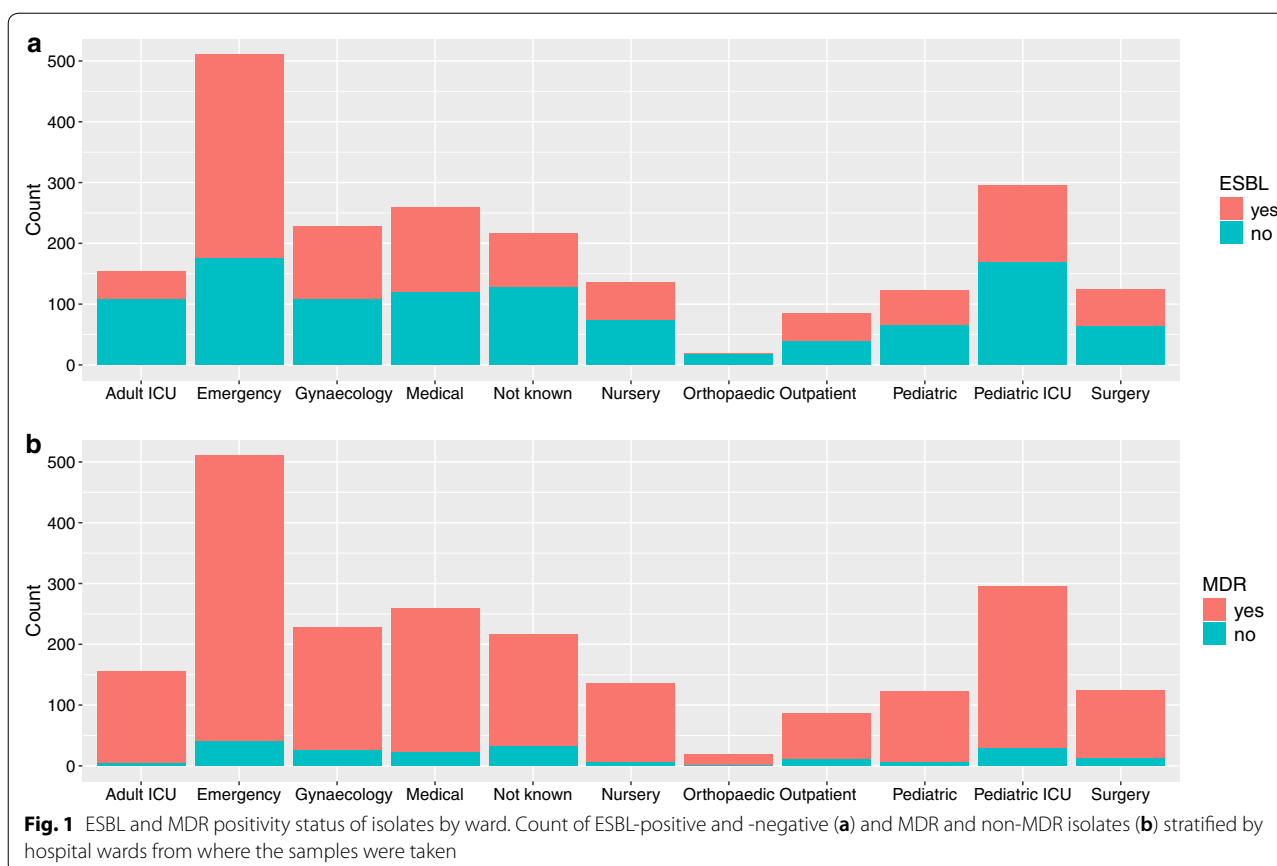


Table 2 ESBL positivity and MDR phenotype stratified by microorganisms

| Microorganisms (N = 2153) | ESBL-neg | | ESBL-pos | | Non-MDR | | MDR | |
|-------------------------------------|----------|------|----------|------|---------|------|-----|------|
| | n | % | n | % | n | % | n | % |
| <i>E. coli</i> (N = 719) | 294 | 40.9 | 425 | 59.1 | 76 | 10.6 | 643 | 89.4 |
| <i>Klebsiella</i> spp. (N = 532) | 210 | 39.5 | 322 | 60.5 | 44 | 8.3 | 488 | 91.7 |
| <i>Enterobacter</i> spp. (N = 520) | 189 | 36.3 | 331 | 63.7 | 10 | 1.9 | 510 | 98.1 |
| <i>Acinetobacter</i> spp. (N = 382) | 380 | 99.5 | 2 | 0.5 | 68 | 17.8 | 314 | 82.2 |

The distribution of AMR genes

The presence of screened AMR genes, stratified by the number of microorganisms tested is presented in Table 3. In *E. coli* isolates, 90.1% (648/719) were positive for *bla*_{TEM}, 58.0% (417/719) were positive for *bla*_{CTXM-8} and 37.4% (269/719) were positive for *bla*_{OXA}. In the *Enterobacter* spp., 56.2% (292/520) were positive for *bla*_{OXA}, 59.6% (201/520) were positive for *bla*_{KPC}, 80.3% (417/520) were positive for *bla*_{CTXM-1} and 57.9% (301/520) were positive for *bla*_{TEM}. In *Klebsiella* spp., 77.4% (408/532) were positive for *bla*_{CTXM-1}, 67.2% (357/532) were positive for *bla*_{OXA}, 4.1% (22/532) were positive for *bla*_{KPC} and 62.5% (332/532) of tested isolates were positive for

*bla*_{TEM}. In *Acinetobacter* spp., 58.6% (218/382) were positive for *bla*_{OXA51}, 36.0% (134/382) were positive for *bla*_{OXA23} and 20.7% (79/382) were positive for *bla*_{NDM-1}.

The association of ESBL genes with ESBL phenotypes

For all subsequent analysis, isolates were classified as *bla*_{OXA} or *bla*_{CTXM} positive if any gene in the *bla*_{OXA} or *bla*_{CTXM} gene families were detected respectively. First, we assessed the association between phenotypic ESBL positivity and the presence of *bla*_{OXA}, *bla*_{CTXM}, and *bla*_{TEM} (Table 4). The detection of each of these genes was significantly associated with an ESBL phenotype. The strength of association was variable between different

Table 3 Summary of detection of AMR genes

| Organisms | Genes | CTX-M1 | | CTX-M2 | | CTX-M8 | | CTX-M9 | | CTX-M25 | | | | | |
|----------------------|-------------------|--------|----------------|-----------|----------------|--------|----------------|--------|----------------|---------|----------------|-------|----------------|-------|----------------|
| | | n | % ^a | n | % ^a | n | % ^a | n | % ^a | n | % ^a | | | | |
| <i>E. coli</i> | neg | 612 | 85.4 | 717 | 100 | 302 | 42.0 | 714 | 99.3 | 710 | 98.7 | | | | |
| | pos | 105 | 14.6 | 0 | 0 | 417 | 58.0 | 5 | 0.7 | 9 | 1.3 | | | | |
| | n.t. ^b | 2 | | 2 | | 0 | | 0 | | 0 | | | | | |
| <i>Enterobacter</i> | neg | 102 | 19.7 | 0 | n.a | 0 | n.a | 518 | 99.8 | 0 | n.a | | | | |
| | pos | 417 | 80.3 | 0 | n.a | 0 | n.a | 1 | 0.2 | 0 | n.a | | | | |
| | n.t. ^b | 1 | | 520 | | 520 | | 1 | | 520 | | | | | |
| <i>Klebsiella</i> | neg | 119 | 22.6 | 527 | 100 | 525 | 99.6 | 524 | 99.4 | 521 | 98.9 | | | | |
| | pos | 408 | 77.4 | 0 | 0 | 2 | 0.4 | 3 | 0.6 | 6 | 1.1 | | | | |
| | n.t. ^b | 5 | | 5 | | 5 | | 5 | | 5 | | | | | |
| <i>Acinetobacter</i> | neg | 368 | 98.9 | 372 | 100 | 372 | 100 | 372 | 100.0 | 372 | 100 | | | | |
| | pos | 4 | 1.1 | 0 | 0 | 0 | 0 | 0 | 0.0 | 0 | 0 | | | | |
| | n.t. ^b | 10 | | 10 | | 10 | | 10 | | 10 | | | | | |
| Organisms | Genes | OXA | | OXA1_4_30 | | OXA23 | | OXA24 | | OXA48 | | OXA51 | | OXA58 | |
| | | n | % ^a | n | % ^a | n | % ^a | n | % ^a | n | % ^a | n | % ^a | n | % ^a |
| <i>E. coli</i> | neg | 450 | 62.6 | 0 | n.a | 0 | n.a | 0 | n.a | 715 | 99.4 | 0 | n.a | 0 | n.a |
| | pos | 269 | 37.4 | 0 | n.a | 0 | n.a | 0 | n.a | 4 | 0.6 | 0 | n.a | 0 | n.a |
| | n.t. ^b | 0 | | 719 | | 719 | | 719 | | 0 | | 719 | | 719 | |
| <i>Enterobacter</i> | neg | 228 | 43.8 | 0 | n.a | 0 | n.a | 0 | n.a | 221 | 65.6 | 0 | n.a | 0 | n.a |
| | pos | 292 | 56.2 | 0 | n.a | 0 | n.a | 0 | n.a | 116 | 34.4 | 0 | n.a | 0 | n.a |
| | n.t. ^b | 0 | | 520 | | 520 | | 520 | | | | 520 | | 520 | |
| <i>Klebsiella</i> | neg | 174 | 32.8 | 0 | n.a | 0 | n.a | 0 | n.a | 475 | 89.5 | 0 | n.a | 0 | n.a |
| | pos | 357 | 67.2 | 0 | n.a | 0 | n.a | 0 | n.a | 56 | 10.5 | 0 | n.a | 0 | n.a |
| | n.t. ^b | 1 | | 532 | | 532 | | 532 | | 1 | | 532 | | 532 | |
| <i>Acinetobacter</i> | neg | 0 | n.a | 342 | 91.9 | 238 | 64.0 | 363 | 97.6 | 0 | n.a | 154 | 41.4 | 357 | 96.0 |
| | pos | 0 | n.a | 30 | 8.1 | 134 | 36.0 | 9 | 2.4 | 0 | n.a | 218 | 58.6 | 15 | 4.0 |
| | n.t. ^b | 382 | | 10 | | 10 | | 10 | | 382 | | 10 | | 10 | |
| Organisms | Genes | IMP | | KPC | | NDM1 | | SHV | | TEM | | VIM | | | |
| | | n | % ^a | n | % ^a | n | % ^a | n | % ^a | n | % ^a | n | % ^a | | |
| <i>E. coli</i> | neg | 577 | 99.7 | 719 | 100 | 681 | 94.7 | 719 | 100 | 71 | 9.9 | 578 | 99.8 | | |
| | pos | 2 | 0.3 | 0 | 0.0 | 38 | 5.3 | 0 | | 648 | 90.1 | 1 | 0.2 | | |
| | n.t. ^b | 140 | | 0 | | 0 | | 0 | | 0 | | 140 | | | |
| <i>Enterobacter</i> | neg | 90 | 100 | 136 | 40.4 | 412 | 79.2 | 515 | 99.0 | 219 | 42.1 | 90 | 100 | | |
| | pos | 0 | 0 | 201 | 59.6 | 108 | 20.8 | 5 | 1.0 | 301 | 57.9 | 0 | 0.0 | | |
| | n.t. ^b | 183 | | 183 | | 0 | | 0 | | 0 | | 430 | | | |
| <i>Klebsiella</i> | neg | 100 | 100 | 509 | 95.9 | 414 | 78.0 | 415 | 78.2 | 199 | 37.5 | 100 | 100 | | |
| | pos | 0 | 0 | 22 | 4.1 | 117 | 22.0 | 116 | 21.8 | 332 | 62.5 | 0 | 0.0 | | |
| | n.t. ^b | | | 1 | | 1 | | 1 | | 1 | | 432 | | | |
| <i>Acinetobacter</i> | neg | 0 | n.a | 0 | n.a | 303 | 79.3 | 361 | 97.0 | 300 | 78.5 | 0 | n.a | | |
| | pos | 0 | n.a | 0 | n.a | 79 | 20.7 | 11 | 3.0 | 82 | 21.5 | 0 | n.a | | |
| | n.t. ^b | 382 | | 382 | | 90 | | 10 | | 90 | | 382 | | | |

^a Percentage of positive or negative in all isolates tested for a particular gene^b Not tested

Table 4 Association between ESBL positivity, MDR or non-susceptibility to cephalosporins and presence of resistance genes

| Genes | ESBL | non-ESBL | OR (95% CI) ^c | p-value ^d |
|---|-----------------------------|-------------------------|--------------------------|----------------------|
| <i>bla</i> _{OXA} ^a | | | | |
| OXA-neg | 406 | 458 | | |
| OXA-pos | 674 | 604 | 1.3 (1.1–1.5) | 0.009 |
| <i>bla</i> _{CTXM} ^b | | | | |
| CTXM-neg | 112 | 660 | | |
| CTXM-pos | 968 | 401 | 14.2 (11.2–18.1) | < 0.001 |
| <i>bla</i> _{TEM} | | | | |
| TEM-neg | 262 | 517 | | |
| TEM-pos | 818 | 545 | 3.0 (2.5–3.6) | < 0.001 |
| Genes | MDR | non-MDR | OR (95% CI) ^c | p-value ^d |
| <i>bla</i> _{OXA} ^a | | | | |
| OXA-neg | 751 | 113 | | |
| OXA-pos | 1198 | 80 | 2.3 (1.7–3.1) | < 0.001 |
| <i>bla</i> _{CTXM} ^b | | | | |
| CTXM-neg | 629 | 143 | | |
| CTXM-pos | 1319 | 50 | 6.0 (4.2–8.6) | < 0.001 |
| <i>bla</i> _{TEM} | | | | |
| TEM-neg | 680 | 99 | | |
| TEM-pos | 1269 | 94 | 2.0 (1.4–2.7) | < 0.001 |
| Genes | Cephal. 3/4 non-susceptible | Cephal. 3/4 susceptible | OR (95% CI) ^c | p-value ^d |
| <i>bla</i> _{OXA} ^a | | | | |
| OXA-neg | 825 | 35 | | |
| OXA-pos | 1244 | 26 | 2.0 (1.2–3.5) | 0.008 |
| <i>bla</i> _{CTXM} ^b | | | | |
| CTXM-neg | 710 | 54 | | |
| CTXM-pos | 1358 | 7 | 14.7 (6.6–38.6) | < 0.001 |
| <i>bla</i> _{TEM} | | | | |
| TEM-neg | 746 | 27 | | |
| TEM-pos | 1323 | 34 | 1.4 (0.8–2.4) | 0.22 |

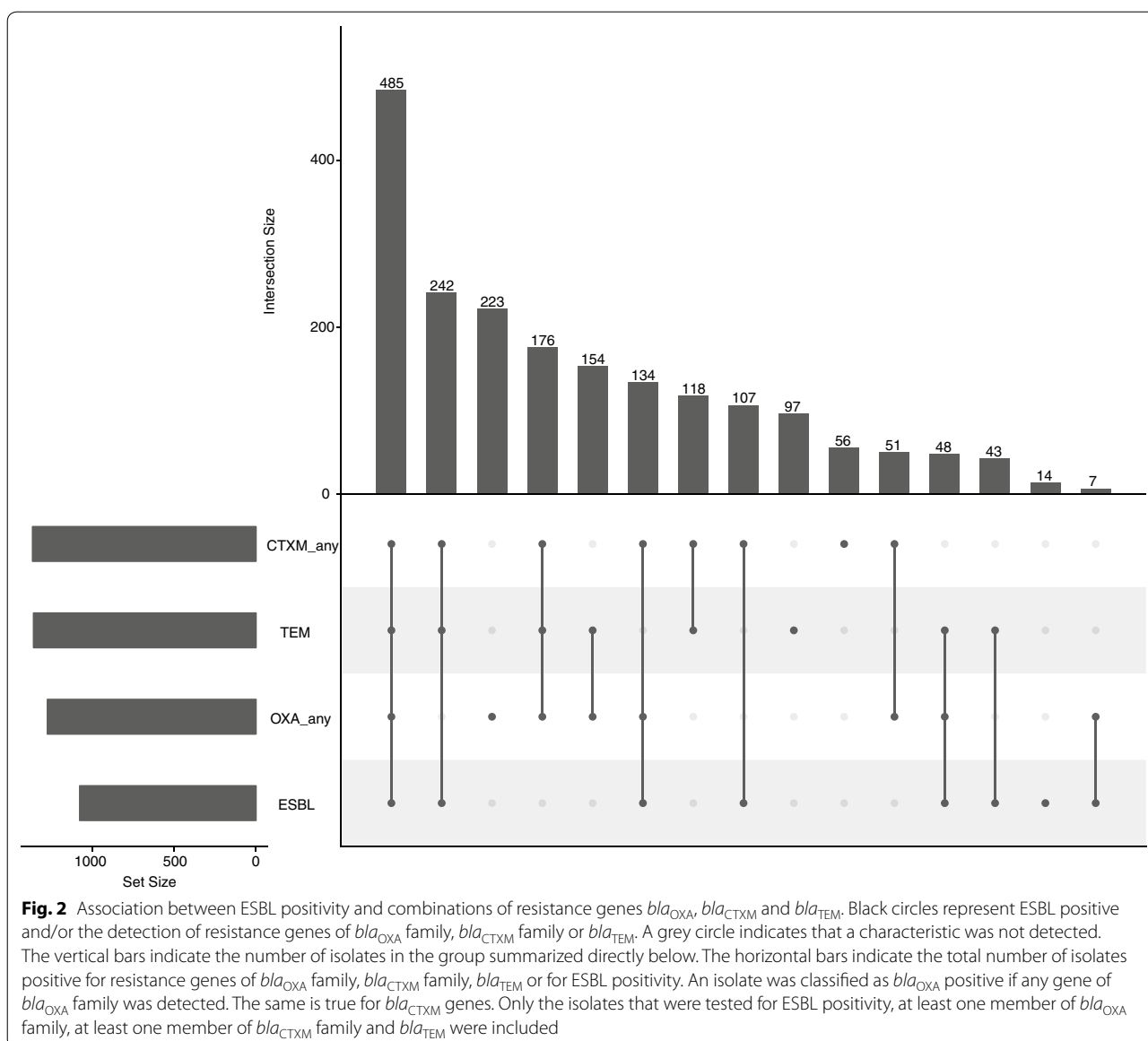
Cephal. 3/4, 3rd or 4th generation cephalosporin

^a An isolate was classified as *bla*_{OXA} positive if *bla*_{OXA1_4_30}, *bla*_{OXA}, *bla*_{OXA-23}, *bla*_{OXA-24}, *bla*_{OXA-48}, *bla*_{OXA-51} or *bla*_{OXA-58} was detected^b An isolate was classified as *bla*_{CTXM} positive if *bla*_{CTXM-1}, *bla*_{CTXM-2}, *bla*_{CTXM-8}, *bla*_{CTXM-9} or *bla*_{CTXM-25} was detected^c Odds ratio (OR and 95% CI) of bacterial isolate to be ESBL positive, multi-drug resistant or resistant to 3rd or 4th generation cephalosporin in the presence of a respective gene (gene family)^d p-value from Fisher's exact test

classes with an odds ratio (OR) of 1.3 (95% CI 1.1–1.5, $p < 0.05$) for *bla*_{OXA}, 2.96 (95% CI 2.5–3.6, $p < 0.001$) for *bla*_{TEM} and 14.2 (95% CI 11.2–18.1, $p < 0.001$) for *bla*_{CTXM} in Fisher's test.

Next, the association between ESBL positivity and various combinations of *bla*_{OXA}, *bla*_{CTXM} and *bla*_{TEM} resistance genes were investigated. The isolates that were tested for at least one member of *bla*_{OXA} family, one member of *bla*_{CTXM} family and *bla*_{TEM} were analysed. A distribution of different combinations of *bla*_{OXA}, *bla*_{CTXM} and *bla*_{TEM} resistance genes with

ESBL positivity is shown in Fig. 2. The most frequent (485/1955; 24.8%) combination of genes that was associated with ESBL positivity was at least one member of *bla*_{OXA} family gene, with at least a member of *bla*_{CTXM} family gene and *bla*_{TEM} gene. A smaller proportion of isolates (176/1955; 9.0%) was PCR positive for *bla*_{OXA}, *bla*_{CTXM} and *bla*_{TEM}, but was phenotypically ESBL negative. The second most common (242/1955; 12.3%) gene combination associated with ESBL positivity was *bla*_{CTXM} with *bla*_{TEM}. The proportion of isolates that were phenotypically ESBL positive, but PCR



amplification negative for *bla*_{OXA}, *bla*_{CTXM} and *bla*_{TEM} was 0.7% (14/1955).

The association of ESBL genes with non-susceptibility to third and fourth generation cephalosporins

We also tested for the association between non-susceptibility to third and fourth generation cephalosporins (Cefotaxime, Cefepime, Ceftriaxone and Cefixime) and the presence of *bla*_{OXA}, *bla*_{CTXM}, or *bla*_{TEM} (Table 4). The *bla*_{CTXM} and *bla*_{OXA} gene families were significantly associated with non-susceptibility to third and fourth generation cephalosporins with odds ratios of 14.7 (95% CI 6.6–38.6, $p < 0.001$) and 2.03 (95% CI 1.2–3.5, $p < 0.05$) respectively. The most frequent combination (654/2121;

30.8%) associated with non-susceptibility against third and fourth generation cephalosporins was the presence of all three *bla*_{OXA}, *bla*_{CTXM}, and *bla*_{TEM} genes, followed by the presence of *bla*_{CTXM} and *bla*_{TEM} only (357/2121; 16.8%). Only 8.9% (189/2121) of isolates that were phenotypically non-susceptible to third and fourth generation cephalosporins were PCR amplification negative for *bla*_{OXA}, *bla*_{CTXM}, and *bla*_{TEM} (Fig. 3).

The association of ESBL genes with MDR

We similarly investigated for any potential association between the presence of *bla*_{OXA}, *bla*_{CTXM} or *bla*_{TEM} genes with MDR (Table 4). A multi-drug resistant phenotype was significantly associated with the presence of *bla*_{CTXM}

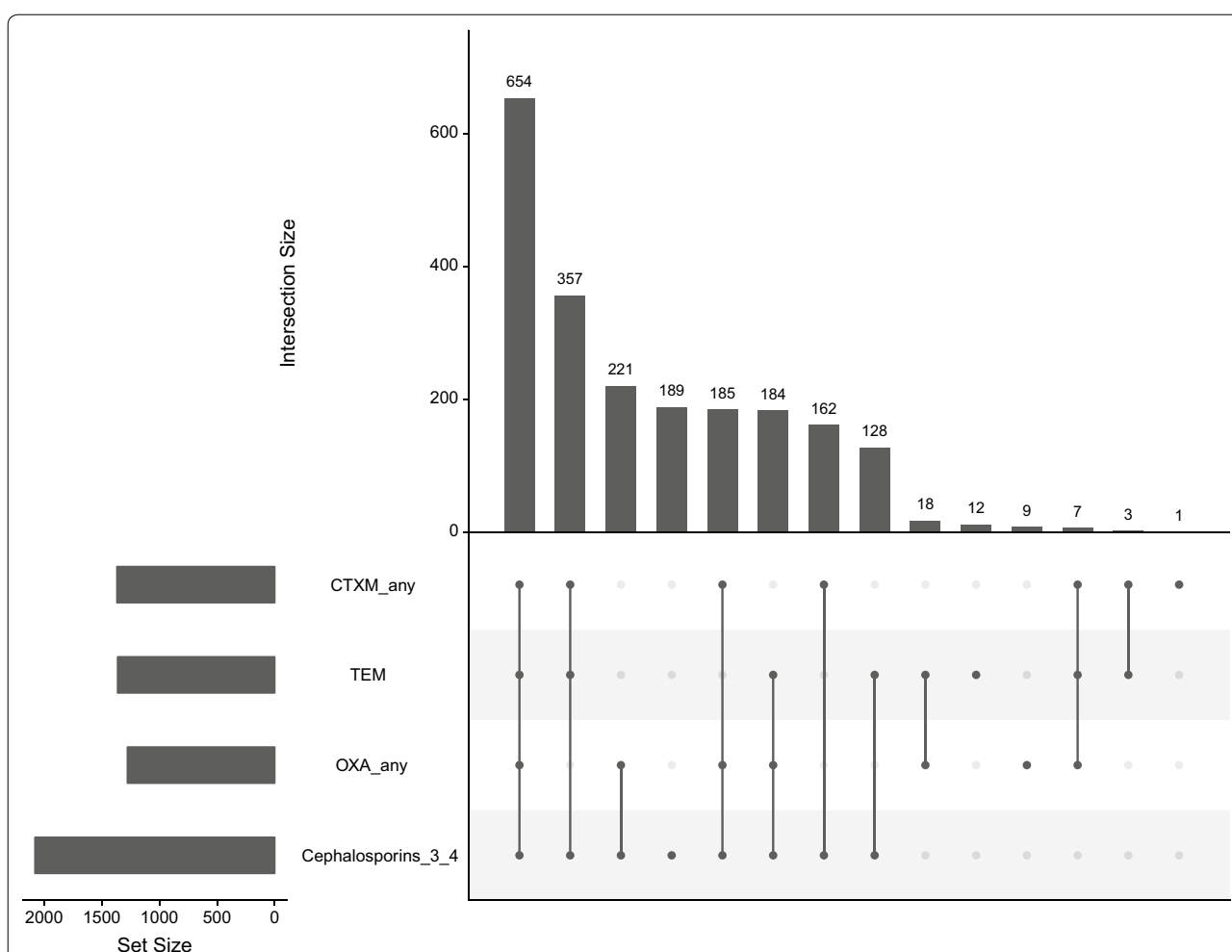
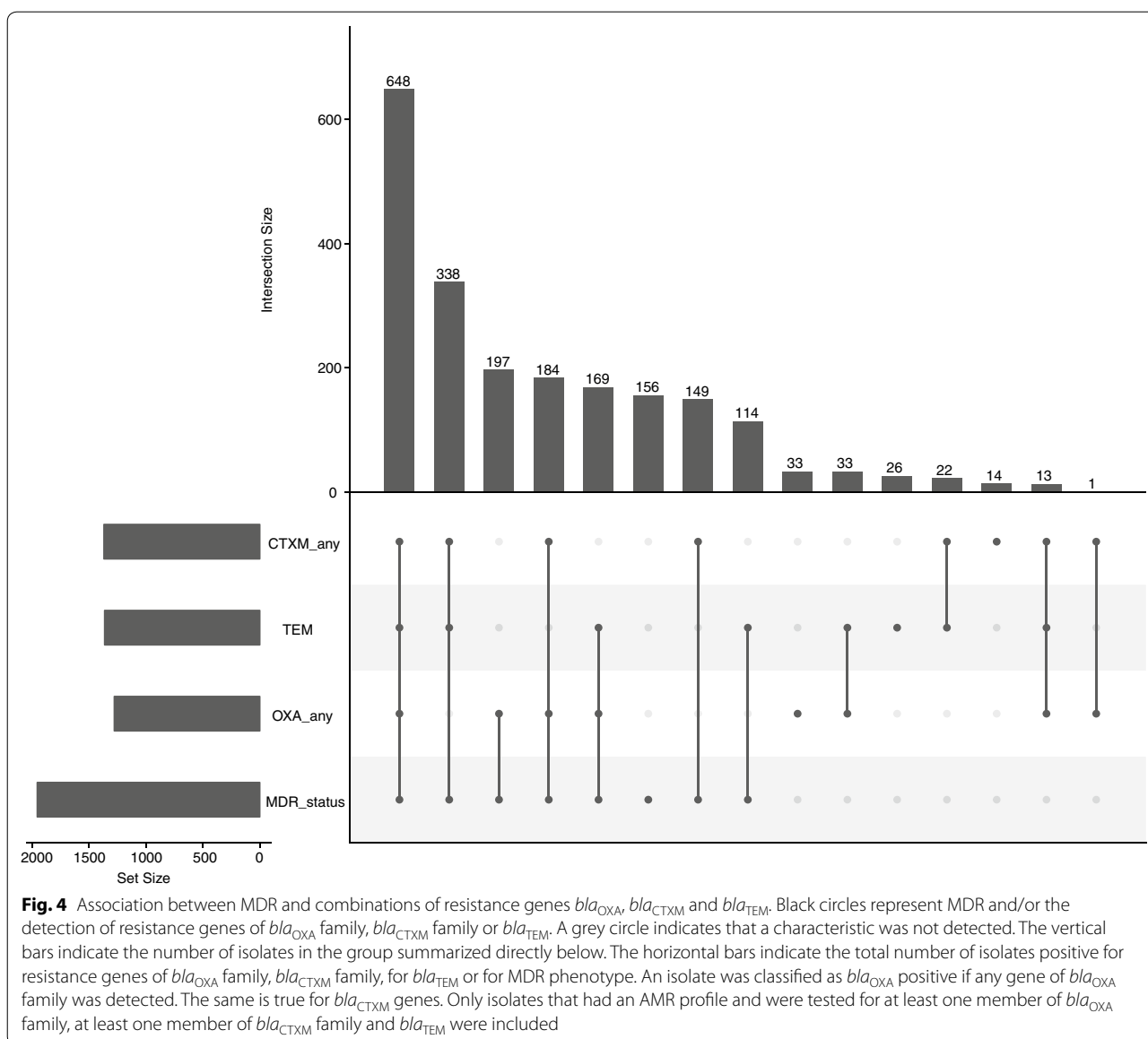


Fig. 3 Association between non-susceptibility to 3rd and 4th generation cephalosporins and combinations of resistance genes *bla*_{OXA}, *bla*_{CTXM} and *bla*_{TEM}. Black circles represent non-susceptibility to cephalosporins (3rd/4th gen.) and/or the detection of resistance genes of *bla*_{OXA} family, *bla*_{CTXM} family or *bla*_{TEM}. A grey circle indicates that a characteristic was not detected. The vertical bars indicate the number of isolates in the group summarized directly below. The horizontal bars indicate the total number of isolates positive for resistance genes of *bla*_{OXA} family, *bla*_{CTXM} family, for *bla*_{TEM} or for non-susceptibility to 3rd and 4th generation cephalosporins. An isolate was classified as *bla*_{OXA} positive if any gene of *bla*_{OXA} family was detected. The same is true for *bla*_{CTXM} genes. Only isolates that had a known 3rd and 4th generation cephalosporin resistance profile and were tested for at least one member of *bla*_{OXA} family, at least one member of *bla*_{CTXM} family and *bla*_{TEM} were included

(OR 5.99, 95% CI 4.2–8.6, $p < 0.001$), *bla*_{OXA} (OR 2.3, 95% CI 1.7–3.1, $p < 0.001$) and *bla*_{TEM} (OR 1.96, 95% CI 1.4–2.7, $p < 0.001$). Additionally, the most common gene combinations associated with MDR were *bla*_{OXA} and *bla*_{CTXM} with *bla*_{TEM} genes (648/2097; 30.9%), followed by *bla*_{CTXM} with *bla*_{TEM} (338/2097; 16.1%). A small fraction of isolates (156/2097; 7.4%) were PCR amplification negative for *bla*_{OXA}, *bla*_{CTXM} and *bla*_{TEM}, but were multi-drug resistant phenotypes (Fig. 4).

As MDR was defined as an acquired non-susceptibility to at least one agent in three different classes of antimicrobials, a multi-drug resistant phenotype can originate from a multitude of individual antimicrobial

resistant phenotypes. Therefore, we compared the pattern of resistance for the isolates originating from outpatients (emergency ward and outpatient department, Fig. 5a) and that from inpatients (all other wards, Fig. 5b). For the microorganisms originating from the outpatients, the three most frequent AMR combinations accounted for over half (322/535; 60.2%) of all outpatient isolates. For the microorganisms originating from the inpatients, the three most frequent AMR combinations accounted only for 26% of all inpatient isolates (282/1057) and the diversity of resistant phenotypes was much broader than that from outpatient isolates.



Discussion

In this study, very high prevalence of ESBL positivity and MDR was found in Enterobacteriaceae family bacteria (*E. coli*, *Klebsiella* spp. and *Enterobacter* spp.). Similar MDR prevalence has been reported in other studies from Nepal, but the ESBL positive prevalence we observed (over 50%) is higher than others have reported. For example, one study from Nepal found that in community-acquired infections caused by *E. coli*, 24% were ESBL positive and 78% had MDR [12]. Another hospital-based study from Nepal showed that in urine samples, 27% of *E. coli* were ESBL positive and 97% of Enterobacteriaceae bacteria (mostly *E. coli* and *Klebsiella* spp.) were multi-drug resistant [13]. For *Acinetobacter* spp., we report

over 81% MDR, similar to what others have reported in ICUs in Nepal, but the ESBL prevalence we found (<1% in *Acinetobacter* spp.) is much lower than what has been reported (around 12–13%) [15, 16, 29]. In the context of South Asia, 79% of *Acinetobacter* spp., 71% of *Klebsiella* spp. and 54% of *E. coli* isolated from young infants were estimated to be multi-drug resistant strains [19]. ESBL positivity was confirmed in 42% of *K. pneumoniae* and 33% of *E. coli* isolated from several hospitals in India [30], 40% in Enterobacteriaceae isolates from Pakistan [18] and 16% in enteric and non-enteric Gram-negative bacilli from Bangladesh [31].

To limit the spread of antimicrobial resistant micro-organisms, to guide clinical care and improve patients'

(See figure on next page.)

Fig. 5 AMR patterns for outpatients and inpatients. AMR patterns are presented for outpatients (emergency and outpatients departments) in (a) and for inpatients (all other hospital wards) in (b). Only the 42 most frequent combinations are shown. Black circles represent resistance to the antimicrobials groups and/or multidrug resistance (MDR). The vertical bars represent the number of isolates characterized by the AMR pattern described under the bar. The horizontal bars indicate the total number of isolates resistant to the different groups, or MDR

outcome, it is essential to have precise knowledge on AMR profile of the infecting bacteria prior to starting antimicrobial therapy to select an appropriate drug. Antimicrobial sensitivity testing and detection of ESBL positivity can be performed by classical phenotypical methods such as disc diffusion, which is labour intensive and time consuming. Automated systems exist, but their installation, running and maintenance costs are often a barrier in low resource settings. Alternatively, simple genetic methods such as PCR can detect AMR genes rapidly and with high sensitivity [2, 10, 32]. A better understanding of the association between phenotype and genotype is a prerequisite to evaluate the usefulness of genetic detection methods in clinical settings. In this study, we assessed the correlation between the detection of *bla*_{CTXM}, *bla*_{TEM} and *bla*_{OXA} genes by PCR and ESBL positivity or MDR as detected phenotypically by disc diffusion.

For *E. coli*, *Enterobacter* spp. and *Klebsiella* spp., the resistance genes most frequently detected were *bla*_{OXA}, *bla*_{TEM}, *bla*_{CTXM-1} and *bla*_{CTXM-8}. This is consistent with a study performed in 2012–2013 at Kathmandu Medical College and Teaching Hospital, in which *bla*_{CTXM} and *bla*_{TEM} were frequently detected in ESBL positive *E. coli* [14]. A study from Bangladesh reported *bla*_{CTXM-1} (51%) and *bla*_{SHV} (27%) to be the most common ESBL genes in *Klebsiella pneumoniae* [17]. In Pakistan and India, as in our study, *bla*_{OXA}, *bla*_{TEM} and *bla*_{CTXM}, in addition to *bla*_{SHV} were detected to be common AMR genes in Enterobacteriaceae isolates [18, 30]. For *Acinetobacter* spp., *bla*_{OXA-51}, *bla*_{OXA-23} and *bla*_{NDM-1} were the most frequently detected AMR genes, as found by others in Nepal, India and Bangladesh [33–35].

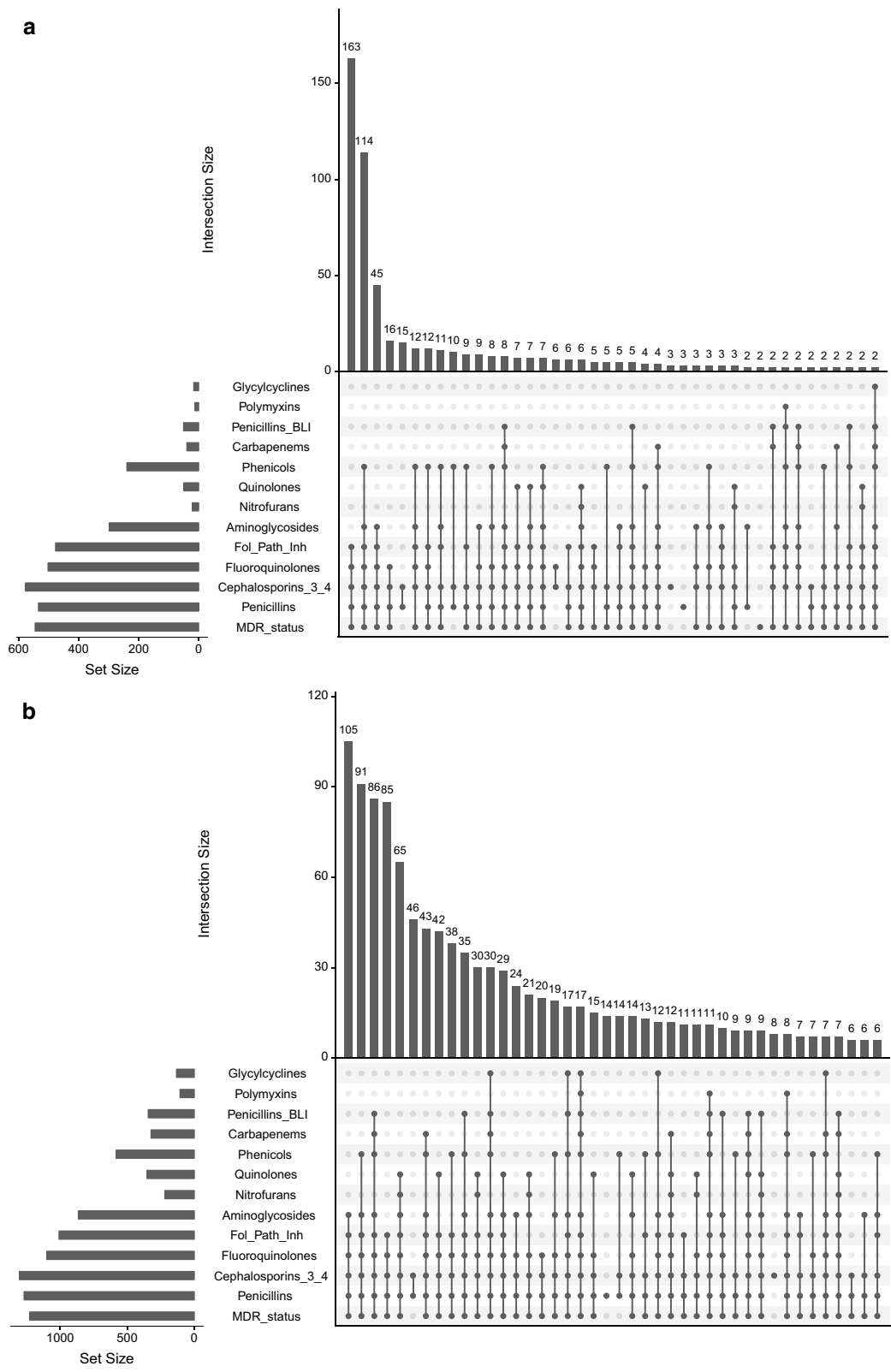
The detection of any gene of the *bla*_{CTXM} family was strongly associated with ESBL positivity (OR 14.2, 95% CI 11.2–18.1), MDR (OR 5.99, 95% CI 4.2–8.6) and resistance to 3rd and 4th generation cephalosporins (OR 14.7, 95% CI 6.6–38.6). The detection of *bla*_{TEM} was strongly associated with ESBL positivity (OR 2.96, 95% CI 2.5–3.6) and MDR (OR 1.96, 95% CI 1.4–2.7). An association between *bla*_{TEM} and resistance to 3rd and 4th generation cephalosporins was suggested by an OR of 1.4, but it was not statistically significant (95% CI 0.8–2.4), perhaps due to low number of isolates susceptible to 3rd and 4th generation cephalosporins. Finally, the detection of any gene of the *bla*_{OXA} family was associated with multi-drug

resistant phenotype (OR 2.3, 95% CI 1.7–3.1). This suggests that the detection of *bla*_{TEM}, or any gene of the *bla*_{CTXM} or *bla*_{OXA} family is an important index for multi-drug resistant phenotype, and as expected, the detection of a *bla*_{CTXM} family gene or *bla*_{TEM} indicates a higher odds of ESBL positive phenotype. Collectively, this suggests that the detection of key AMR genes by molecular methods is an important index for ESBL positivity and MDR in bacterial isolates.

In this study, we also described different AMR patterns found in the isolates originating from emergency and outpatient wards (outpatients) compared to all other wards of the hospital (inpatients), which may reflect different usage of antimicrobials in community compared to the hospital. It can be assumed that isolates originating from outpatients reflect what is circulating in communities. For outpatients, the three most frequent AMR combinations accounted for more than half of all isolates. Perhaps people contracting community-acquired infections predominantly self-medicate, which results in exposure to the limited array of antimicrobials that are freely available in shops and pharmacies. Only infections that are (or have become) resistant to those drugs prompt a visit to the hospital. As a result, infections seen in outpatients have similar resistance profile. In contrast, a higher diversity of AMR combinations found in inpatients may reflect a wider array of antimicrobials available and used at hospitals.

Conclusions

MDR along with possession of ESBL and carbapenemase associated resistance genes among Gram-negative bacilli pose a serious problem in therapeutic management of patients. The compromised infection control and inadequate antimicrobial usage policies coupled with high burden of Gram-negative bacilli possessing transferable antimicrobial resistance genes in resource limited settings set out an ideal scenario for the emergence and dissemination of multi-drug resistant pathogens. In this extensive retrospective study, a high burden of multi-drug resistant clinical Gram-negative bacilli possessing diverse ESBL and carbapenemase resistance *bla* genes were evidenced. Further, our study signifies that there is a high probability of Gram-negative bacilli to be multi-drug resistant and ESBL positive in case of detection of any of *bla*_{TEM}, *bla*_{CTXM} or *bla*_{OXA} family genes.



Additionally, the detection of any of the *bla*_{CTXM} family genes highly implies that the Gram-negative bacilli are non-susceptible to the extended-spectrum beta-lactam antimicrobials.

Supplementary information

Supplementary information accompanies this paper at <https://doi.org/10.1186/s12941-020-00390-y>.

Additional file 1: Table S1. Classes of antimicrobials and their members.

Abbreviations

AMR: Antimicrobial resistance; CI: Confidence interval; CLSI: Clinical and laboratory standards institute; DNA: Deoxy-ribonucleic acid; ESBL: Extended-spectrum beta-lactamase; MDR: Multi-drug resistance; OR: Odds ratio; PCR: Polymerase chain reaction; PICU: Pediatric intensive care unit; WGS: Whole genome sequencing; XDR: Extensive-drug resistance.

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Authors' contributions

AK conceptualized the research and has a major contribution in writing and editing the manuscript. BB performed manuscript editing. GT performed manuscript editing. KGP performed laboratory work and manuscript editing. NM performed statistical analysis of data. RMZ performed statistical analysis of data and preparation of graphs. SB reviewed manuscript and has a major contribution in manuscript editing. SD performed laboratory work and manuscript editing. SM conducted the research, performed laboratory work, data management, data analysis, results interpretation, major manuscript writing, editing and formatting. SMD performed manuscript editing. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analysed during this study are included in this published article and its supplementary information files.

Ethics approval and consent to participate

An ethical approval for conducting this study was taken from Nepal Health Research Council (NHRC) (Reference number 1017). Data used in this retrospective study were the anonymised routine microbiology laboratory results originating from Patan Hospital. These data were devoid of any patients' identifying information. Further, the data used in this study were a part of routine surveillance for infection prevention and control at Patan Hospital. Thus, individual written informed consent was not required.

Consent for publication

All data used in this study were the anonymised microbiological data devoid of patients' identification and personal information. Thus obtaining consent for publication is not applicable for this study. All authors consented for the publication of this research.

Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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